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Approaches to functionally validate candidate genetic variants involved in colorectal cancer predisposition

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ABSTRACT

Most next generation sequencing (NGS) studies identified candidate genetic variants predisposing to colorectal cancer (CRC) but do not tackle its functional interpretation to unequivocally recognize a new hereditary CRC gene. Besides, germline variants in already established hereditary CRC-predisposing genes or somatic variants share the same need when trying to categorize those with relevant significance. Functional genomics approaches have an important role in identifying the causal links between genetic architecture and phenotypes, in order to decipher cellular function in health and disease. Therefore, functional interpretation of identified genetic variants by NGS platforms is now essential. Available approaches nowadays include bioinformatics, cell and molecular biology and animal models. Recent advances, such as the CRISPR-Cas9, ZFN and TALEN systems, have been already used as a powerful tool with this objective. However, the use of cell lines is of limited value due to the CRC heterogeneity and its close interaction with microenvironment. Access to tridimensional cultures or organoids and xenograft models that mimic the in vivo tissue architecture could revolutionize functional analysis. This review will focus on the application of state-of-the-art functional studies to better tackle new genes involved in germline predisposition to this neoplasm.

1. Introduction

As for other complex diseases, colorectal cancer (CRC) is caused by both genetic and environmental factors. Twin studies showed that around 13%–30% of the variation in CRC susceptibility involves inherited genetic differences (Lichtenstein et al., 2000; Frank et al., 2017). Some of the known CRC-predisposing factors were already discovered in the past decade (Peters et al., 2015). Next generation sequencing (NGS) has revolutionized our ability to read information from the genome, including the DNA sequence itself, the state of the transcriptome and the epigenome, among others (Casey et al., 2013). NGS has tremendously improved the identification of disease candidate genetic variants. However, most NGS studies did not tackle its functional interpretation of these variants to unequivocally recognize a new hereditary CRC gene (Valle, 2017). Also, similarly to the germline counterpart, the same functional interpretation difficulty is encountered when somatic studies are pursued to identify clinically relevant variants (Ng et al., 2018). Functional interpretation of the identified variants involved in germline predisposition and somatic studies is essential to establish an unambiguous link to disease predisposition or progression. Available approaches nowadays include bioinformatics, cell and molecular biology and animal models. Recent advances, such as the CRISPR-Cas9 system, have already showed high potential to be used in this direction (Mali et al., 2013a; Komor et al., 2017).

Functional studies of genetic variants suspected of being involved in predisposition to disease are still scarce in most research or hospital centers where NGS technologies are applied. However, they are imperative for the correct interpretation of results in the fields of research and clinical diagnosis. Aim to generalize and facilitate this type of

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studies, tools have been recently developed that allow carrying them out massively and in parallel (Wei et al., 2014).

Functional genomics approaches have an important role in identifying the causal links between genetic architecture and phenotypes, in order to decipher cellular function in health and disease. Therefore, functional interpretation of genetic variants identified by NGS platforms is now essential. It should be mentioned that the use of cell lines is of limited value due to the CRC heterogeneity and its close interaction with microenvironment. Therefore, access to tridimensional (3D) cultures and xenograft models that mimic the in vivo tissue architecture may revolutionize functional analysis (Ribatti, 2014; Dutta et al., 2017).

On the other hand, the emergence of CRISPR/Cas9 technology is allowing a much more fluid modification of the genome at the time to introduce the genetic variants to be studied and for its subsequent functional dissection in cellular models. Moreover, 3D models permit culture and outgrowth of intestinal crypt cells into organoids (Dutta et al., 2017), recapitulating the physiology, shape, dynamics and cell make-up of the intestinal epithelium (Grabinger et al., 2014). In addition, the chorioallantoic membrane assay is a highly reproducible xenograft model (Ribatti, 2014). Such models play an important role in the screening and evaluation of new biomarkers and their functional characterization. Besides, genes of interest can be knocked out and their functional effects on the gut can be studied (Mizutani et al., 2017).

The present chapter aims at reviewing the current knowledge and available tools to better characterize the functional effect of genetic variation. Particularly, it will focus on the application of state-of-the-art functional studies to better tackle genes involved in germline predisposition to CRC.

2. Bioinformatics

The use of exome and genome sequencing to unveil new variants involved in disease genetics faces a relentless bottleneck when variant identification studies stumble upon large lists of candidate variants waiting to be prioritized (Cline and Karchin, 2011). Prior to functional studies, and due to time-consume and economical high costs of these experiments, variant impact potentiality must be assessed (Quintáns et al., 2014; Tang and Thomas, 2016). Multiple bioinformatics tools to approach this issue have been developed, enabling annotation, scoring and classification of variants to comprehensively estimate the deleteriousness of human genetic variants (Niroula and Vihinen, 2016). Specially, single nucleotide variants (SNVs) at coding regions have been the main focus when developing tools to predict a functional impact, being rare non-synonymous variants intrinsically more plausible diseases candidates (Cooper and Shendure, 2011; Li et al., 2013).

Prioritization and prediction tools assume that disease may arise through a change in protein's amino acid sequence and collaterally affecting its function (Mooney et al., 2010; Tang and Thomas, 2016). Thus, prediction tools may use different perspectives to approach this premise: (1) direct protein structure inspection, where variant effect is predicted from its possible effects on protein stability and function; or (2) sequence comparison studying either nucleotide sequence or amino acid sequences from different species, reflecting the effects of negative natural selection by noticing those positions that display evolutionary conservation among homologs (Cline and Karchin, 2011). However, protein structure availability is mandatory for those structure-based methods. Therefore, sequence conservation-based tools have been used extensively and great efforts have been made to improve these methods, arising multiple tools during the last two decades (Tang and Thomas, 2016). Table 1 summarizes bioinformatics tools available for pathogenicity prediction of genetic variants. Renowned methods such as SIFT (Stone and Sidow, 2005), PANTHER (Thomas et al., 2003), polyPhen2 (Adzhubei et al., 2010), GERP (Cooper et al., 2005) or LRT (Chun and Fay, 2009) have been extensively applied in variant identification and prioritization studies (Gylfe et al., 2013; Tanskanen et al., 2015; Esteban-Jurado et al., 2015). Moreover, meta-prediction tools trying to integrate outcomes from prediction tools have also been developed. Examples are CONDEL (González-Pérez and López-Bigas, 2011), CAROL (Lopes et al., 2012), CoNVEC (Frousios et al., 2013) or CADD (Kircher et al., 2014; Rentzsch et al., 2018).

On the other hand, RNA splicing excise introns and splice exons. It is essential that splicing sites are correctly recognized and, indeed, some germline mutations involved in human disease predisposition alter this process. In order to test a putative splicing alteration, either analysis of the RNA patient or in silico prediction tools are also used (Jian et al., 2014).

Huge amount of variants identified by NGS studies cannot be handled roughly. Therefore, reliable tools are needed to prioritize those potential deleterious variants into functional validation studies. However, the wealth of pathogenicity prediction tools in the literature could imply unknown divergences between methods, and implications on this behalf shall be assessed (Cooper and Shendure, 2011). Studies to find out prediction tools accuracy when applied to wide datasets or to specific gene variants also have been performed (Mahmood et al., 2017; Grimm et al., 2015; Frousios et al., 2013). Unfortunately, these bioinformatics tools do not offer a perfect solution for variant prioritization and most researchers use several of them to reinforce their final candidate variant list. Benchmarking and optimization of all these prediction methods will be essential to characterize the tremendous number of future variants that owe to be identified.

3. Gene editing

In recent years, the CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats) gene editing technology has transformed functional genomics, enabling researchers to potentially edit any desired region of the genome. The molecular complex, based on the adaptive defense system of bacteria and archaea, consists of two elements: an RNA sequence (guide RNA, gRNA) complementary to the target DNA, and a nuclease (Cas) that recognizes the RNA/DNA hybrid and performs a specific double-strand break (DSB) on it. Upon cleavage, the target locus can be repaired through the error-prone non-homologous end joining (NHEJ) pathway or by homologous recombination (HR), which requires a DNA donor template. Hence, DNA repair through NHEJ forces insertions and deletions, thereby inactivating the target gene, while HR allows introducing specific changes in the genome (Ran et al., 2013).

With this basis, the CRISPR/Cas system has been deeply explored and adapted to become a versatile laboratory tool. Cas9, the most commonly used nuclease, has been single (Cas9 nickase) and double mutated (Cas9-null or dead-Cas9, dCas9) to improve its specificity and targeting functions. The nickase performs single strand breaks due to a unique active catalytic domain, and has a reduced off-target activity as well as a higher in-situ editing efficiency (Komor et al., 2017). By contrast, dCas9 has no endonuclease activity but can be specifically targeted to any dsDNA sequence due to the gRNA/Cas9 interaction. This CRISPR strategy, which is referred to as CRISPR interference (CRISPRi) (Qi et al., 2013), can be used to recruit transcriptional activators and inhibitors to regulatory zones, to modulate epigenetic marks and even to modify the genome architecture (Mali et al., 2013a). Moreover, there are other Cas proteins with different protospacer adjacent motifs (PAM) requirements, which increases targeting possibilities (Rath et al., 2015). Its adaptability and enzymatic improvements make the CRISPR system an efficient alternative to other functional genomics approaches. Gene silencing by siRNAs and shRNAs is a fast and inexpensive method, but generates an incomplete and temporary gene inactivation, has unpredictable off-target effects and shows poor reproducibility. Other gene editing strategies, such as TALEN (Transcription Activator-Like Effector Nucleases) and ZFN (Zinc-finger nucleases), perform accurate gene modifications but require a new nuclease design, synthesis, and validation for each target DNA (Gaj et al.,

Table 1
Tools for variant deleteriousness prediction at the protein level. Bioinformatics tools for variant deleteriousness prediction by analyzing sequence conservation and phylogenic information. Prediction tools based on nucleotide sequence or amino acid sequence are specified. Meta-prediction tools integrating multiple predictions are also detailed. Analysis source, web links and reference for each prediction tool are listed.

Name	Prediction by	Analysis based on	Web link	References
phastCons	Nucleotide sequence	Phylogenetic conservation	http://compgen.cshl.edu/phast/help-pages/phastCons.txt	Siepel et al. (2005)
GERP		Phylogenetic conservation	http://mendel.stanford.edu/SidowLab/downloads/gerp/	Cooper et al. (2005)
phyloP		Phylogenetic conservation (includes GERP)	http://compgen.cshl.edu/phast/help-pages/phyloP.txt	Pollard et al. (2010)
SCONE		Phylogenetic conservation	http://genetics.bwh.harvard.edu/scone/	Asthana et al. (2007)
VISTA		Phylogenetic conservation	http://genome.lbl.gov/vista/	Dubchak et al. (2000); Frazer et al. (2004)
MAPP	Amino acid sequence	Phylogenetic conservation and biochemical features	http://mendel.stanford.edu/SidowLab/downloads/MAPP/	Stone and Sidow (2005)
SIFT		Phylogenetic conservation and biochemical features	http://sift.bii.a-star.edu.sg/	Ng and Henikoff (2001)
PANTHER		Phylogenetic conservation and biochemical features	http://www.pantherdb.org/	Thomas et al. (2003)
MutationTaster*		Phylogenetic conservation, biochemical and structural features	http://www.mutationtaster.org/	Schwarz et al. (2010)
nsSNP Analyzer		Phylogenetic conservation, biochemical and structural features	http://snpanalyzer.uthsc.edu/	Bao et al. (2005)
PMUT		Phylogenetic conservation, biochemical and structural features	http://mmb.pcb.ub.es/PMut	Ferrer-Costa et al. (2004)
polyPhen		Phylogenetic conservation, biochemical and structural features	http://genetics.bwh.harvard.edu./pph2/	Adzhubei et al. (2010)
SNAP		Phylogenetic conservation, biochemical and structural	http://www.rostlab.org/services/SNAP/	Bromberg and Rost (2007)
SNPs3D		Phylogenetic conservation, biochemical and structural	http://www.snps3d.org/	Yue et al. (2005)
PhD-SNP		Phylogenetic conservation and biochemical features	http://gpcr2.biocomp.unibo.it/~emidio/PhD-SNP/PhD-SNP_Help.html	Capriotti et al. (2006)
LRT		Phylogenetic conservation	www.genetics.wustl.edu/jflab/lrt_query.html	Chun and Fay (2009)
FATHMM		Phylogenetic conservation	http://fathmm.biocompute.org.uk	Shihab et al. (2013b)
MutationAssessor		Phylogenetic conservation	http://mutationassessor.orgF	Shihab et al. (2013a), 2013b
CONDEL	Meta-tool	MutationAssessor + FATHMM (last release)	https://bbglab.irbbarcelona.org/tools/condel	González-Pérez and López-Bigas, 2011
CAROL		SIFT + Polyphen2	https://www.sanger.ac.uk/science/tools/carol	Lopes et al. (2012)
CADD		phastCons + phyloP + GERP + SIFT + PolyPhen2	https://cadd.gs.washington.edu	Kircher et al. (2014); Rentzsch et al. (2018)
CoVEC		SIFT + PANTHER + PolyPhen2 + MutationAssessor + CONDEL + PhD-SNP + SNPs&GO (3D structure information)	http://sourceforge.net/projects/covec/files	Frousios et al. (2013)

GERP: Genomic Evolutionary Rate Profiling; SCONE: Sequence CONservation Evaluation; MAPP: Multivariate Analysis of Protein Polymorphism; SIFT: Sorting Intolerant From Toloerant; PANTHER: Protein ANalysis THrough Evolutionary Relationships; PolyPhen-2: Polymorphism Phenotyping v2; PhD-SNP: Predictor of human Deleterious Single Nucleotide Polymorphisms; LRT: Likelihood Ratio Test; FATHMM: Functional Analysis through Hidden Markov Models; CONDEL: Consensus Deleteriousness Score; CAROL: Combined Annotation scoRing toOL; CADD: Combined Annotation Dependent Depletion; CoVEC: Consensus Variant Effect Classification).

2013).

CRC modeling by CRISPR/Cas has allowed characterizing the hereditary genes involved in the development of the disease, as well as the somatic mutational events. In CRC cell lines, gene editing has confirmed the effect of several point mutations in the proofreading domain of *POLE* (Van Gool et al., 2018), the enhanced sensitivity to MEK inhibitors of homozygous $KRAS^{G13D}$ mutants (Burgess et al., 2017), as well as the role of *MLH1*, whose in vitro inactivation recreated a hypermutated profile (Germano et al., 2017). Non-coding RNAs involved in CRC malignancy have also been CRISPR-inactivated, such as miR30-a (Shen et al., 2017), CCAT2 (Yu et al., 2017) and CYTOR (Wang et al., 2018), the latter by a precise whole-exon deletion. Furthermore, CRISPR enhancer disruption (Cohen et al., 2017), transcriptional repressor recruitment by dCas9 (Zhang et al., 2018) and multiplexed loss-of-function screenings of epigenetic regulators (McCleland et al., 2016) have allowed the identification of new therapeutic targets.

Several studies have already applied CRISPR/Cas in CRC organoids to determine the pathogenicity of the most well-known mutations, as well as to reconstruct the adenoma-carcinoma sequence. For instance, the combination of gene disruption and precise gene editing strategies has allowed generating quadruple ($KRAS^{G12D}/APC^{KO}/P53^{KO}/SMAD4^{KO}$) and quintuple ($KRAS^{G12V}/PI3KCA^{E545K}/APC^{KO}/P53^{KO}/SMAD4^{KO}$) CRISPR-mutated CRC organoids (Drost et al., 2015; Fumagalli et al., 2017; Matano et al., 2015). Mutations can also be added to those already existing in the patients' organoids. The CRC serrated pathway has been reproduced in vitro by sequentially introducing inactivation mutations in five different genes (*TGFBR2*, *RNF43*, *ZNRF3*, *CDKN2A*, *MLH1*) on $BRAF^{V600E}$ organoids (Lannagan et al., 2018). CRISPR has also been employed to enhance CRC organoid generation by marking adult intestinal stem cells with Enhanced Green Fluorescent Protein (EGFP), bypassing the lack of good commercial antibodies to detect Leucine-rich repeat-containing G-protein coupled receptor 5+ (LGR5+) cells (Cortina et al., 2017).

All these studies have demonstrated the power of CRISPR/Cas for studying the functional consequences of genomic alterations in CRC. Nevertheless, this genome editing system has not been much used to study germline mutations in new candidate predisposition genes. Either by gene inactivation or single base editing, CRISPR/Cas modeling could be the key to decipher the pathogenicity of many variants of unknown significance. For example, the CRISPR inactivation of *NTHL1* in organoids has been able to reproduce the mutational signature 30, previously observed in breast cancer patients with *NTHL1* germline variants (Drost et al., 2017) Additionally, high-throughput methods to validate SNVs are also being developed, combining gene editing in cancer cell lines coupled with molecular functional assays (Coggins et al., 2017).

Before the CRISPR craze took over the genome editing field with the announcement of the 2015 Breakthrough of the Year, zinc finger nucleases (ZFN) and transcription activator like effector nucleases (TALEN) were declared the "Method of the Year" in 2011 (Anonymous, 2012; Travis, 2015). The popularity of the CRISPR/Cas9 system stems from the ease with which constructs expressing guide RNA's and the Cas9 gene can be assembled. Yet there may still be some biotechnological or therapeutic use for ZFN and TALEN. The ability of all three programmable nucleases to cleave off-target sequences raise questions about therapeutic safety (Pattanayak et al., 2013). The difficulty of experimentally detecting off-target specificity because of the inherent error rate of sequencing technology prevents an accurate comparison of the different technologies (Akcakaya et al., 2018).

Recently the efficiency of genome editing by CRISPR/Cas9 was linked to p53 mediated stress responses and cell cycle arrest (Haapaniemi et al., 2018; Ihry et al., 2018). These studies showed that in stem cells and in certain cell lines, p53 deficiency conferred an advantage in CRISPR/Cas9 mediated genome editing. This finding could limit the editing of stem cell genomes because of the potential for selecting p53 deficient clones that may have a higher risk of oncogenesis. Whether ZFN and TALEN are also affected by the absence of p53 is not yet clear. What is surprising is that a single CRISPR/Cas9 induced double strand break in the genome can result in cell cycle arrest. It is possible that occupancy of the cut site by different programmable nucleases may induce different mechanisms of repair.

Delivery methods often limit the transfer of genome editing nucleases to primary cells. In cases where gene transfer is not desired, RNA or protein transfection may be a possibility. In the case of TALEN, alternative direct delivery of proteins to target cells has been demonstrated (Liu et al., 2014). With these limitations and potential advantages in mind, it is useful to revisit the now "old" mechanisms of ZFN and TALEN mediated genome editing.

ZFN are biotechnological tools that started the genome editing craze. These are chimeric nucleases that bind to DNA using several zinc finger motifs (Kim et al., 1996). A comparison of transcriptional activator families in eukaryotes shows that in many species the zinc finger family makes up the largest group (Tupler et al., 2001). Because zinc finger DNA binding domains were the most common in nature, they were the best choice to construct a chimeric nuclease. The crystal structure of zinc finger motifs shows that these motifs contact DNA by inserting a helix into the major groove and make four contacts, three in the top strand and one in the bottom strand (Pavletich and Pabo, 1993). A zinc atom coordinates this helix and two beta strands in each zinc finger motif. Artificial ZFN typically fuse 3-4 N-terminal zinc finger motifs to a C-terminal nuclease domain derived from the *Fok*I restriction endonuclease. *Fok*I is a Type IIS restriction enzyme that recognizes a non-palindromic site (GGATGN9/CCTACN13) and cleaves outside of its recognition sequence. ZFN only contain the DNA cleaving domain of the enzyme and not its DNA binding domain. DNA binding specificity of a ZFN is purely directed by the zinc finger motifs. While zinc fingers tend to recognize G-rich targets, a direct code that links amino acid sequences in the DNA-recognizing alpha helix and the bound bases does not exist. In fact, the specificity of the three bases recognized by each motif is position and context dependent. As such, a zinc finger motif that specifically binds to a three base pair sequence in the first position of a four motif DNA binding domain does not necessarily bind to the same sequence if it were present in the position corresponding to the binding site of the second, third or fourth motif. Because of these limitations, ZFN need to be assembled using complicated selection procedures that requires the assembly of each ZFN in case by case fashion (Maeder et al., 2009).

ZFN cut DNA when a heterodimer binds two target sequences flanking a spacer sequence that gets cleaved by the *Fok*I homodimer. The optimum spacer length for a ZFN pair is 5 base-pairs. Various optimizations were made that prevent the formation of homodimers and only result in the formation of heterodimers (Szczepek et al., 2007; Miller et al., 2007). An early demonstration of a successful ZFN was a study targeting human stem cells (Hockemeyer et al., 2009). ZFN-based genome editing is not dead and gone. Several candidates are still in the pipeline of biotechnology companies such as Sangamo Therapeutics as of the last quarter of 2019.

TALEN are also dimers of site specific nucleases that use the *Fok*I restriction domain (Sanjana et al., 2012). The development of TALEN is based on the identification of the DNA binding domain of the AvrBs3/PthA or TAL (transcription activator-like) family proteins expressed in the plant pathogenic bacteria belonging to the Xanthomonas spp. (Boch and Bonas, 2010). Breaking the code of TALE DNA recognition was an important finding that catapulted these tools into the limelight of genome editing (Boch et al., 2009). Crystal structures demonstrating how TALE proteins grab DNA was also a very exciting finding that revealed a novel DNA binding mode for transcription factors (Mak et al., 2012; Deng et al., 2012; Gao et al., 2012). 2011 was a year of revolutions for the field of genome editing. The development of many tools to assemble TALEN rapidly and efficiently shifted the interest of many researchers in the field from ZFN to TALEN (Hockemeyer et al., 2011; Miller et al., 2011; Cermak et al., 2011). Unfortunately, TALEN were

only in the limelight for a short period of time, losing their popularity to CRISPR by 2013 (Mali et al., 2013b; Ran et al., 2013).

Double strand breaks generated by either CRISPR/Cas9, ZFN or TALEN result in a cellular DNA damage response that initiates the repair of the cut site either by the error prone mechanism of NHEJ or HR if donor DNAs are provided (Bibikova et al., 2001, 2003). While CRISPR/Cas9 is easy to engineer, ZFN and TALEN may still be useful in specifically recognizing and modifying target DNA sequences. A critical advantage of TALEN may rely on the unusual helical structure of the protein. DNA binding protein helices may find use as nanotechnological carriers. Moreover, chimeric proteins that fuse alternative functional domains to either ZFN or TALEN may still yield an advantage over CRISPR/Cas9 (Guha and Edgell, 2017).

4. Model systems - patient-derived organoids

Functional studies based on already established cancer cell lines have been shown to be insufficient and not representative of the heterogeneity and behavior of CRC in patients. Cell lines are exposed to a passage-dependent accumulation of mutations and an extensive clonal selection (Hidalgo et al., 2014; Pillai and Uthamanthil, 2017). Moreover, most cell-line based studies lack normal tissue-derived cells as a reference and do not consider the impact of the stroma (Drost and Clevers, 2018). In recent years, patient-oriented models have emerged to fill the gap between cancer genetics and molecular diagnostics, enabling more personalized approaches (van de Wetering et al., 2015; Weeber et al., 2017).

CRC organoids are self-organized 3D cell culture systems mainly established from human colonic epithelium stem cells or induced pluripotent stem cells (iPSC). They are propagated in vitro using different combinations of growth factors like Wnt/R-spondin1, EGF, Noggin, ALK 4/5/7 and p38 inhibitors (Ohta and Sato, 2014; Sato et al., 2011; Weeber et al., 2017). They preserve the in vivo structure and genetic background of the original tissue, including their mutational profile (Drost and Clevers, 2018), copy-number alterations (CNA) and indels (Jung et al., 2011; Weeber et al., 2015), and the methylation pattern (Roerink et al., 2018), that remain stable over time.

Patient-derived organoids (PDO) generation starts from cell suspensions, which make possible to model organoids with genetic heterogeneity, clonal organoids or even genetically-modified organoids by CRISPR technologies. These strategies can be used for modeling CRC genetics and depict cancer initiation and progression, as well as intratumor heterogeneity and diversification (Roerink et al., 2018). Several studies have generated PDO harboring *APC*, *P53, SMAD4*, and *KRAS* somatic mutations (Drost et al., 2015; Matano et al., 2015; Weeber et al., 2015). Organoids with germline mutations in CRC hereditary genes have also been developed, like *APC* (Crespo et al., 2017), *MUTYH* (Lo et al., 2017) and *MLH1*, even recreating the mutational signature of mismatch repair-deficient CRCs (Drost et al., 2017). However, it seems that genetically engineered organoids do not fully mimic chromosomal aberrations and DNA methylation patterns of the original tumors (Salahudeen and Kuo, 2015).

Upon in vitro culturing and maintenance, organoids can also be xenotransplanted in mice, either CRC-derived organoids (Roper et al., 2018; Vlachogiannis et al., 2018) or genetically modified organoids (Drost et al., 2015; Fumagalli et al., 2017; Matano et al., 2015). They have been successfully implanted subcutaneously, orthotopically or in the kidney capsule. Since they can be genetically edited, CRC PDO models are the gold standard to study the genetic spectrum and mutational processes that drive malignant transformation. They have also been employed for drug screenings as they closely recapitulate patient responses (Pauli et al., 2017; Vlachogiannis et al., 2018).

5. Model systems - patient-derived xenografts

Patient-derived xenografts (PDX) models consist of the implantation of cancerous cell suspensions or small pieces of tumors derived from primary surgical resection into immunocompromised mice, with subsequent re-engraftment to successive generations. CRC PDXs are a very reliable and precise approximation to the human tumor counterpart, as genetic alterations are usually concordant with the parental tumor (Bertotti et al., 2011; Julien et al., 2012; Seol et al., 2014) and are maintained upon sequential passage (Kreso et al., 2013), including mutations in classically CRC-associated genes, as well as CNA (Bertotti et al., 2011) and small indels. Some PDX unique mutations have also been detected, most of them related with the stroma components rather than tumor cells, probably due to the coexistence and progressive replacement of human stroma by the murine one. Gene expression and protein expression patterns are also well preserved, as well as the histological structure, the lymphatic system and blood vasculature (Julien et al., 2012). By now, the application of PDX models has been mainly focused on drug discovery and personalized chemotherapy trials through the so-called Avatar mice (Aparicio et al., 2015). Some studies have revealed a surprising correlation between the response to chemotherapy of CRC patients and the response of their corresponding PDXs, as well as associations between gene mutations and drug resistance (Bertotti et al., 2011; Hidalgo et al., 2014; Migliardi et al., 2012).

PDO and PDX are cutting-edge tools that can be used to characterize new CRC susceptibility genes. PDO are an easier tool to develop and gene editing is affordable, although mutations affecting tumor-stroma interactions are not reflected. On the other hand, PDX seem to better resemble CRC tumors (Schütte et al., 2017) but imply high resource consumption, and cancer initiation approaches are difficult. There is a great need for setting up PDO and PDX biobanks to integrate the existing cohorts and establish new correlations between genetic markers and drug sensitivity (van de Wetering et al., 2015), as well as to depict the clinical heterogeneity of human CRC (Brown et al., 2016). Some of the initiatives are the OncoTrack consortium (http://www.oncotrack.eu) (Schütte et al., 2017), the Hubrecht Organoid Technology (HUB) living biobank (http://hub4organoids.eu/), and the EurOPDX consortium (https://europdx.eu/). Although considering PDO and PDX as a clinical prognostic tool is still premature, the integration of these models with NGS can help to monitor the genomic changes that trigger CRC development and allow personalized therapy designs. Fig. 1 aims to be a schematic of the different approaches described in the previous sections.

6. Functional validation of genetic variants in CRC – hereditary genes

The identification of pathogenic variants in genes predisposing to CRC allows the clinical diagnosis of hereditary CRC syndromes. Aiming to get a classification with clear clinical impact, integration of multiple lines of evidence is needed, and the obtained by using functional assays must be among them. Functional assays can evaluate the impact of a variant at different molecular levels. In this section, we will briefly review the main tools that have been used for the functional validation of germline variants in genes predisposing to hereditary CRC.

At RNA level, genetic variants can affect transcription levels, mRNA splicing or transcript stability. In silico predictions are usually used to identify variants that can potentially alter the mRNA splicing (Jian et al., 2014). Technical validation of these predictions includes assays such as cDNA analysis in tissues from variant carriers and minigenes (Castellsagué et al., 2010; Gaildrat et al., 2010, Borras et al., 2013).

Functional assays at the protein level are important in the evaluation of the pathogenicity of a variant, provided that the evaluated property is relevant to the mechanism of pathogenicity of the gene in that disease (Richards et al., 2015; Nykamp et al., 2017). A variant can affect the protein at different molecular steps (e.g. expression and stability, subcellular localization, complex formation, specific function) (Fig. 2). To address the impact on the protein function of a hereditary

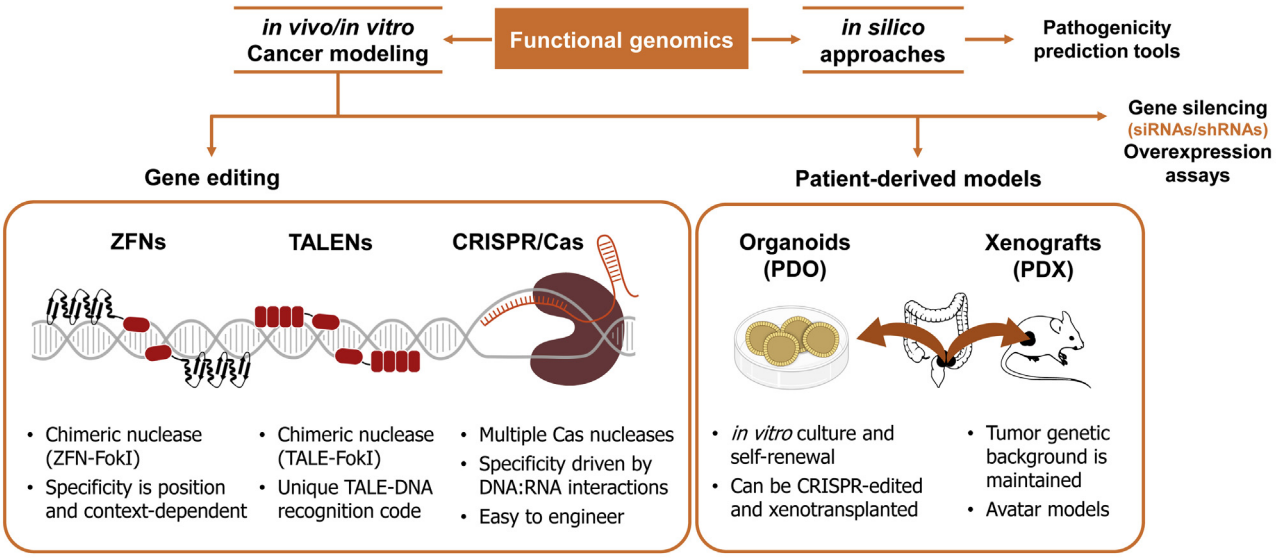

Fig. 1. In vivo, in vitro and in silico approaches in functional genomics. In silico methodologies include pathogenicity bioinformatics prediction tools, whereas in vivo/in vitro cancer modeling ranges from gene silencing and overexpression preliminary assays to more complex approaches such as gene editing and patient-derived models.

CRC gene variant, gene-specific assays have been developed. It is important to consider whether the assay has been conducted under physiological conditions, if the experimental model is human, if the function of the complete protein is evaluated, and if results are concordant between different laboratories. Here, the main experimental approaches are reviewed.

Mismatch repair genes: Mismatch repair (MMR) system corrects base-base mismatches and small insertion/deletions mainly introduced by DNA polymerases during replication, but also mispairs formed during recombination or chemically modified bases (Reyes et al., 2015). Within the large diversity of assays used in functional assessment of MMR variants, methods addressed to evaluate the MMR capability,

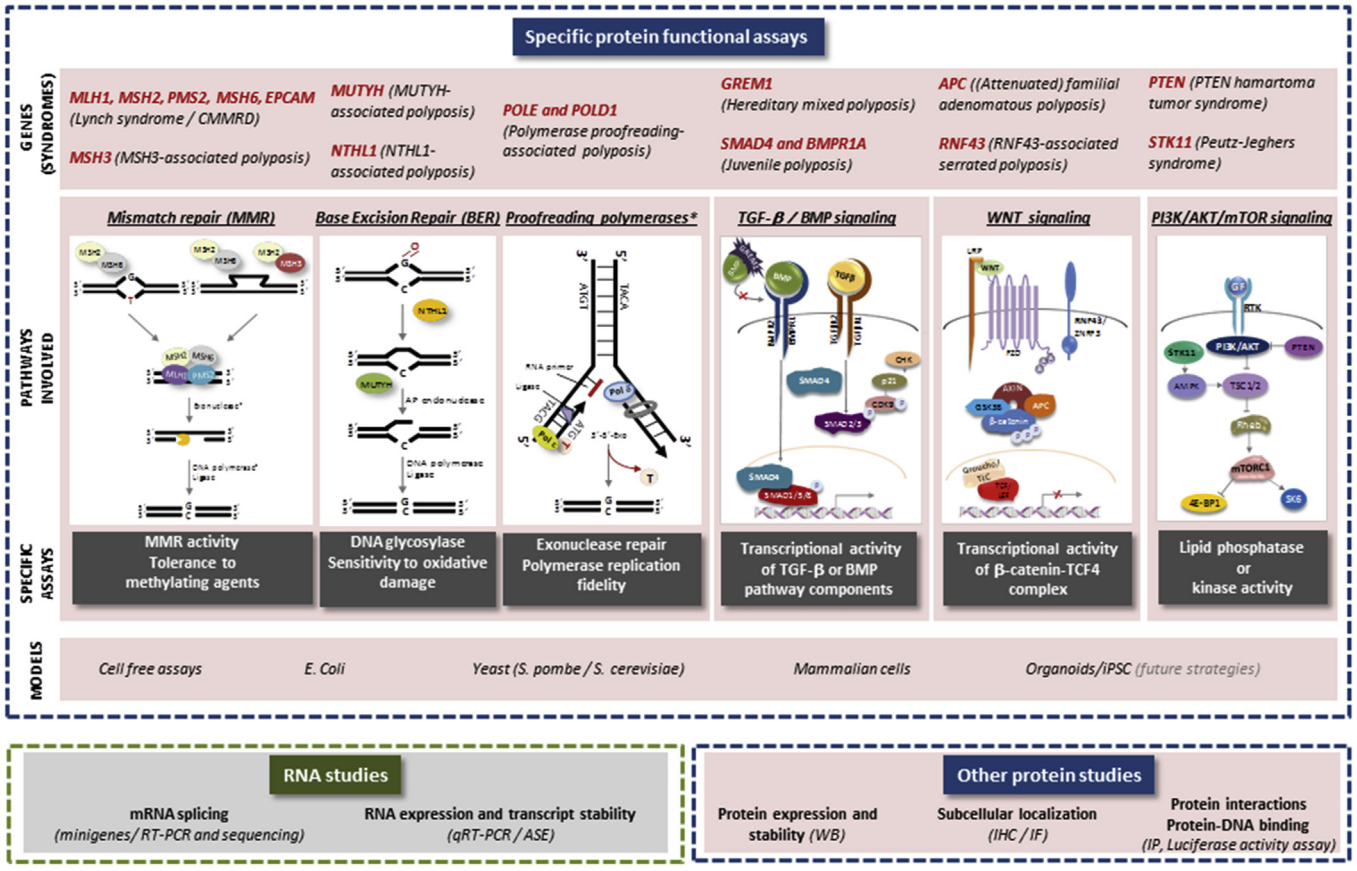

Fig. 2. Functional testing of hereditary CRC gene variants. RT-PCR, reverse transcription PCR; qRT-PCR, quantitative real time PCR; WB, western blot; IHC, immunohistochemistry; IF, immunofluorescence; IP, immunoprecipitation; ASE, allele-specific expression.

likely the most important function of a MMR protein, are proposed as the gold standard to study MMR variants (Borràs et al., 2012; Hinrichsen et al., 2015; Peña-Diaz and Rasmussen, 2016). In vitro cell-free assays, consisting in a reconstitution assay using cell-free protein extracts or human purified proteins together with nuclei extracts and a substrate to repair, are currently the most commonly used (Plotz et al., 2006; Ollila et al., 2008; Pineda et al., 2015; Drost et al., 2018), giving advantages over former yeast-based in vivo or cell-based ex vivo assays (Peña-Diaz and Rasmussen, 2016). Of note, recently a cell-free in vitro MMR activity assay has been calibrated and validated, enabling its integration with in silico and clinical data in multifactorial likelihood calculations of pathogenicity (Drost et al., 2018). Protein expression, subcellular localization, heterodimer formation, sensitivity to methylating agents, DNA mismatch binding or ATP processing have also been assessed (Guerrette et al., 1998; Heinen et al., 2002; Ollila et al., 2008; Borras et al., 2013; Hinrichsen et al., 2015).

MUTYH and *NTHL1*: *MUTYH* and *NTHL1* are DNA glycosylases involved in the base excision repair (BER) pathway, which is responsible for the correction of base errors that are caused by oxidative damage, alkylation, deamination or uracil misincorporation (Weren et al., 2015). Whereas *MUTYH* is able of excising mispaired bases, *NTHL1* has both glycosylase and lyase activity. Thus, evaluation in vitro or in vivo of DNA glycosylase activity in different models systems (*E. coli*, yeast, human cell lines) has proven useful to assess functional impact of *MUTYH* and *NTHL1* variants (Yang et al., 2001; Shi et al., 2006; Turco et al., 2013; Brinkmeyer and David, 2015; Komine et al., 2015; Robey-Bond et al., 2017; Limpose et al., 2018). Besides, DNA binding affinity, protein interactions and enzyme kinetics have also been evaluated (D'Agostino et al., 2010; Kundu et al., 2010; Turco et al., 2013; Brinkmeyer and David, 2015; Robey-Bond et al., 2017).

POLE and *POLD1*: Germline variants in *POLE/POLD1* polymerases linked to CRC predisposition are located within the exonuclease domain, affecting the 3′-5′ exonuclease (proof-reading) activity (Briggs and Tomlinson, 2013; Palles et al., 2013; Nicolas et al., 2016). Due to the high homology of the exonuclease domain of *POLE* and *POLD1* in human and yeast, most of the functional assays to evaluate variant exonuclease repair activity are performed in yeast models (*S. cerevisiae* or *S. pombe*) (Palles et al., 2013; Shinbrot et al., 2014; Barbari et al., 2018; Castellsagué et al., 2018). The mutator phenotype is measured as the mutation rate to canavanine resistance or amino acid-defective yeast revertants (Mansour et al., 2001; Northam et al., 2010; Palles et al., 2013; Kane and Shcherbakova, 2014; Barbari and Shcherbakova, 2017; Esteban-Jurado et al., 2017). Unfortunately, such yeast in vivo assays are limited to variants in conserved residues of the exonuclease domain. In addition, in vitro cell-free assays have been developed by assessing the replication fidelity of purified proteins using lacZ forward mutation assay or exonuclease repair activity (Ghodgaonkar et al., 2014; Shinbrot et al., 2014).

APC and *RNF43*: *APC* gene codifies for a multifunctional protein that controls beta-catenin turnover in the Wnt signaling pathway. One of the most studied APC functions is the transcriptional activity mediated by β-catenin-TCF-4 complex using a luciferase reporter assays (Korinek et al., 1997; Azzopardi et al., 2008; Menendez et al., 2008). Subcellular localization, either of APC or its binding proteins can also be interrogated in transfected cells by immunostaining, immunofluorescence or western blot after subcellular fractionation (Kohler et al. 2008, 2009; Menendez et al., 2008). Other functions such as cell migration and adhesion, apoptosis and protein interactions have also been evaluated (Faux et al., 2004; Dikovskaya et al., 2007; Menendez et al., 2008; Harris and Nelson, 2010).

RING finger protein 43 (*RNF43*) is an E3 ubiquitin ligase that inhibit Wnt signaling by interacting with the Wnt receptors of the Frizzled family (Loregger et al., 2015). In order to determine the effect of *RNF43* variants on protein function, luciferase reporter assays have been used in CRC cell lines transfected with *RNF43* variants (Quintana et al., 2018).

BMPR1A, *SMAD4* and *GREM1*: *BMPR1A* and *SMAD4* are genes associated with the TGF-β/BMP signal pathway (Cichy et al., 2014). In this pathway, bone morphogenetic proteins (BMP) bind to BMPR1A, which then dimerizes the receptor and leads to a phosphorylation cascade. This phosphorylation cascade involves the phosphorylation of SMAD proteins that then associate with oligomers of SMAD4, migrate into the nucleus, and associate with DNA-binding proteins. SMAD4-related gene expression leads to changes in genes important in cell growth, differentiation, and apoptosis. Functional analysis of *BMPR1A* and *SMAD4* variants mainly assess the effect on signaling with either BMP responsive element or SMAD luciferase reporter assays in transfected cell lines (Kotzsch et al., 2008; Carr et al., 2012; Howe et al., 2013). Subcellular localization, DNA binding, BMP-2 binding and protein stability -among others-have also been analyzed (Morén et al., 2003; Kuang and Chen, 2004; Kotzsch et al., 2008; Howe et al., 2013). In contrast, no gene-specific functional assays have been reported for *GREM1* variants except for monitoring its increased expression due to the detected promoter duplication.

PTEN: *PTEN* gene encodes for a phosphatase that is essential for regulating diverse biological processes, and through its lipid phosphatase activity regulates the phosphoinositide 3-Kinase/Akt/mTOR signaling pathway (Pilarski et al., 2013). Phosphatase assays are employed to study the catalytic activity of PTEN against phospholipid substrates (Rodríguez-Escudero et al., 2011; Spinelli et al., 2015; Spinelli and Leslie, 2015). PTEN stability and subcellular localization in *S. cerevisiae* or mammalian cells have also been explored (Teresi et al., 2007; Mighell et al., 2018; Mingo et al., 2018).

STK11: STK11 is a serine threonine kinase that regulates cell polarity and energy metabolism (Xu et al., 2013). Specific studies such as evaluation of kinase activity, subcelullar localization, transcriptional activity of downstream targets have been analyzed in variant function evaluation studies (Nezu et al., 1999; Ylikorkala et al., 1999; Forcet et al., 2005; Jiang et al., 2018).

Of note, repair pathway-specific mutational signatures and high mutational burden have been recently described in some tumors as a consequence of the underlying repair defect (Alexandrov and Stratton, 2014; Campbell et al., 2017; Drost et al., 2017; Pilati et al., 2017; Viel et al., 2017). Therefore, it would be relevant to explore the value of these somatic characteristics as a functional surrogate in germline variant classification (Walsh et al., 2018).

In conclusion, well-established functional tests are mandatory to support the pathogenicity of genetic variants in hereditary CRC genes. In the last years, standardized classification systems have allowed improving reproducibility and transparency of variant classification (Thompson et al., 2014; Richards et al., 2015; Nykamp et al., 2017).

7. Functional validation of genetic variants in CRC – germline candidate genes

In recent years, many research groups have proposed new candidate genes for germline predisposition to non-affiliated familial CRC. Although the majority of these studies did not tackle their functional interpretation, a small number made experimental approaches to identify new hereditary CRC genes. The aim of this section is to briefly describe which genes have been deeply characterized through functional analysis and which tools have been used. Table 2 summarizes functional studies performed in candidate genes proposed for germline predisposition to this neoplasm.

For those genes that encode catalytic activity domains, enzymatic assays are an appropriate tool to evaluate the alteration of their particular function. One of the first candidate genes studied through functional studies was *EPHB2*. The research group carried out in vitro kinase assays via expression and immunoprecipitation of *EPHB2* variants in a human cancer cell line (Zogopoulos et al., 2008). Similarly, Guda et al. and Evans et al. analyzed *GALNT12* variants through a transferase activity assay using radiolabeled oligosaccharides, in order to define their

Table 2
Functional studies performed in candidate genes proposed for germline predisposition to colorectal cancer.

Function	Functional techniques	Candidate gene/s	Reference
Enzyme activity	In vitro kinase assays	*EPHB2*	Zogopoulos et al. (2008)
	Transferase activity assay	*GALNT12*	Guda et al. (2009); Evans et al. (2018)
	Helicase assays	*WRN*	Arora et al. (2015)
	Chromatin remodeling assay	*ERCC6*	Arora et al. (2015)
Protein-protein interaction	Co-immunoprecipitation assays	*SMAD9; BUB1B; SETD6*	Ngeow et al. (2015); Hahn et al., Martín-Morales et al. (2017)
Protein localization	Immunofluorescence	*BUB1B, BUB1/BUB3*	Hahn et al., Mur et al. (2018); De Voer et al., 2013
Cytogenetic analysis	Aneuploidy study	*BUB1/BUB3*	De Voer et al., 2013; Mur et al. (2018)
	Mitotic checkpoint analysis	*BUB1/BUB3*	Mur et al. (2018)
	Chromosome segregation analysis	*BUB1/BUB3*	Mur et al. (2018)
DNA fragmentation	Comet assay	*WRN, ERCC6*	Arora et al. (2015)
DNA repair	Microsatellite analysis	*MSH3*	Adam et al. (2016)
	Immunocytochemistry of MMR proteins	*MSH3*	Adam et al. (2016)
Apoptosis	TUNEL assay	*UNC5C*	Coissieux et al. (2011)
	Caspase-3 activity assay	*UNC5C*	Coissieux et al. (2011)
	Annexin V staining assay	*BRF1*	Bellido et al. (2018)
Proliferation and cell cycle	7-AAD/BrdU staining and flow cytometry	*SEMA4A; BRF1*	Schulz et al. (2014); Bellido et al. (2018)
	Flow cytometry	*BRF1*	Bellido et al. (2018)
	Clonogenic survival	*BRF1*	Bellido et al. (2018)
	Cell viability assays	*SEMA4A; BRF1*	Schulz et al. (2014); Bellido et al. (2018)
	Yeast growth assay	*BRF1*	Bellido et al. (2018)
Migration	Cell exclusion zone assay	*SEMA4A*	Schulz et al. (2014)

MMR, mismatch repair; Terminal deoxynucleotidyl transferase dUTP nick end labeling, TUNEL; 7-AAD, actinomycin D; BrdU, 5-bromo-2-deoxyuridine.

implication in glycosylation processes (Guda et al., 2009; Evans et al., 2018). Point mutations can also modify protein-protein interaction networks, which at the same time can modify their subcellular localization and the expression of downstream effectors. These features can be assessed by Western Blot, immunofluorescence or co-immunoprecipitation assays, as performed for *SMAD9* (Ngeow et al., 2015), *BUB1B* (Hahn et al., 2008), *SETD6* (Martín-Morales et al., 2017) and *SEMA4A* (Schulz et al., 2014) variants. Genetic instability is another main hallmark of hereditary cancer. The impact of the *BUB1/BUB3* variants on the mitotic checkpoint was analyzed using cytogenetic analysis (De Voer et al., 2013; Mur et al., 2018). *WRN* and *ERCC6* variants were studied through helicase assays and chromatin remodeling assay. Its implication in DNA fragmentation was evaluated using a comet assay (Arora et al., 2015). Adam et al., estimated *MSH3* variants and its implication with genome instability through microsatellite analysis and immunocytochemistry of MMR proteins (Adam et al., 2016). Many tumor suppressor genes are usually involved in cellular growth and programmed cell death. In order to evaluate the effect of *UNC5C* variants on apoptosis, Coissieux and colleagues performed the TUNEL assay, which detects DNA fragmentation together with Caspase-3 activity assay (Coissieux et al., 2011). Seguí et al. studied the role of *FAN1* variants on cell viability in response to mitomycin C by cell counting analysis (Seguí et al., 2015). The effect of *SEMA4A* variants on proliferation was analyzed by BrdU incorporation, and their role on migration by cell exclusion zone assay (Schulz et al., 2014). Finally, Bellido et al. deeply characterized *BRF1* variants and its implication in the apoptosis pathway via Annexin V staining assay. They also carried out cell cycle assays by flow cytometry, clonogenic survival and cell viability assays, and in order to support a low loss-of-function effect, they also performed a dependent yeast growth assay (Bellido et al., 2018).

In conclusion, it is essential to functionally corroborate the effect of a selected candidate variant and its association with CRC. Although there are multiple useful techniques to study the main cellular processes, immunoblot, RT-PCR, immunocytochemistry and co-immunoprecipitation, together with flow cytometry and fluorescence microscopy, are the most used tools due to their extreme versatility. With proper sample preparation, these methods allow the evaluation of the effect of a vast number of genetic variants.

8. Functional validation of genetic variants in CRC – somatic variants

Somatic mutations in CRC have been under scrutiny due to their importance in metastasis location and predicting patient's response to therapies (Lipsyc and Yaeger, 2015). Driver somatic mutations in primary colorectal tumors are mostly found in *KRAS* (predominantly in exon 3) (Andreyev et al., 2001), *BRAF* (Janakiraman et al., 2010), *Wnt* effector genes (i.e. *APC* and *FBXW7*) (Morkel et al., 2015), *TP53* (Janakiraman et al., 2010), *PIK3CA* and *SMAD4* (Cancer Genome Atlas Network, 2012). Gene editing approach has been used to model colonoscopy-based CRC studies, investigating various oncogenic genetic variants, such as mutations in TP53, Wnt, TGFβ or EGFR pathways (Fumagalli et al., 2017). This section is aimed at reviewing some functional studies performed in these driver genes in order to further characterize its alteration when mutated.

KRAS: Gene editing is broadly used to understand the underlying pathways that become crucial for cancer cell survival. For instance, cell growth mediators were recently characterized in *KRAS*-driven tumors via genome-wide CRISPR screening. The genes that are selectively enhanced or inhibited after CRISPR-Cas9 introduction in CRC cells with mutant *KRAS* ($KRAS^{MUT}$) and cells with wild-type *KRAS* ($KRAS^{WT}$) were identified. Metabolic vulnerabilities of $KRAS^{MUT}$ cell lines showed that these cells are highly dependent on redox balance and nucleotide synthesis (Yau et al., 2017). Introducing mutations in *TP53* and *APC* tumor suppressor genes in situ, following orthotopic transplantation of *APC*, *TP53* and *KRAS* mutant colon organoids, has been also used to reproduce the entire spectrum of metastasis and tumor progression in CRC (Roper et al., 2017).

PIK3CA: Mutations affecting *PIK3CA*, the catalytic subunit of PI3K (phosphatidylinositide 3-kinase), have been reported in 10–20% of CRC patients, mostly found in exons 9 and 20 (Ogino et al., 2014). Despite having a minor effect of the overall prognostic of CRC, PIK3CA mutations could be used as predictive biomarkers to manage patients and expect successful response to targeted therapies, e.g. anti-EGFR (Epidermal Growth Factor Receptor) therapy or adjuvant therapy with aspirin (Cathomas, 2014). Oncogenic *PIK3CA* mutations lead to glutamine dependency in CRC cell lines. Glutamine deprivation induced more apoptosis in these *PIK3CA* mutant cell lines (Hao et al., 2016). CRC cells with mutated *PIK3CA* showed attenuation of apoptosis and facilitated tumor invasion (Samuels et al., 2005).

TP53: The *TP53* gene encodes a protein called tumor protein p53 (or

p53) that acts as a tumor suppressor. *TP53* mutation occurs in more than half of CRC cases and mutations in this gene were found to be more important in therapy response among affected patients (Iacopetta, 2003). Mutant *TP53* led to elevated expression of several CRC cancer stem cell markers such as CD44, LGR5, and ALDH (*Wnt* target genes). Mutant p53 gained additional oncogenic functions along with losing its tumor-suppressive function. Chemotherapy resistance in CRC patients with mutated *TP53* is due to ALDH1A1 over-expression that mediates inhibition of apoptosis (Solomon et al., 2018). In addition to *TP53*, *PIK3CA* mutation status also has predictive value for overall survival in late stage CRC patients that undergo chemotherapy (Li et al., 2018).

APC: Adenomatous polyposis coli (APC) also known as deleted in polyposis 2.5 (DP2.5) is a protein that in humans is encoded by the *APC* gene. The APC protein is a negative regulator that controls beta-catenin concentrations and interacts with E-cadherin, which is involved in cell adhesion. *APC* mutations in CRC patients create truncated APC proteins that not only have lost their tumor suppressive function but have also gained other functional properties (similar to dominant negative effect of mutant *TP53*), through affecting Wnt pathway, chromosome instability and DNA repair, cell adhesion, and cell cycle control. These changes were found to lead to CRC initiation and progression (L. Zhang and Shay, 2017). In vitro studies on *APC* mutant cell lines have shown that β-catenin inhibitory domain (CID) in APC determines tumor transformation. USP7 (ubiquitin specific peptidase 7) depletion reverses Wnt activation in *APC* mutant CRC, hence could be a potential target for CRC patients with *APC* mutation (Novellasdemunt et al., 2017).

FBXW7: F-box/WD repeat-containing protein 7 is a protein that in humans is encoded by the *FBXW7* gene. FBXW7 functions as a ubiquitin ligase and regulates a network of important oncoproteins (Davis et al., 2014). Expression of this gene is lost in tumor tissue compared to their adjacent normal tissue in 6–7.5% of CRC patients (Korphaisarn et al., 2017). In vitro studies have shown genetic alteration in cells with suppressed *FBXW7*. Cell proliferation is upregulated in these engineered cells following enhanced expression of MYC and cyclin-E proteins (Iwatsuki et al., 2010). In vitro deletion of *FBXW7* promotes chromosomal instability, and co-deletion of *FBXW7* and *TP53* created highly aggressive, metastatic/invasive phenotype in vivo (Grim et al., 2012).

SMAD4: Sporadic mutations in *SMAD4* gene are present in 2.1%–20% of CRC patients, and are associated with poor prognosis and less progression free survival time (Mehrvarz Sarshekeh et al., 2017). Increased tumor growth and liver metastasis have been reported for *SMAD4* knockdown clones. Elevated Akt phosphorylation in *SMAD4* deficient cell clones resulted in upregulation of anti-apoptotic proteins (Bcl-2, Bcl-w and Survivin) and resistance to 5-fluorouracil-based therapy (B. Zhang et al., 2014).

BRAF: The B-Raf protein is encoded by the proto-oncogene *BRAF*. Mutations in this gene (mostly V600E) appear in 5–10% of metastatic CRC (Korphaisarn and Kopetz, 2016). Cell line studies showed that *BRAF* amplification in *BRAF*-mutated subsets of cells increases MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) phosphorylation, leading to resistance to MEK-inhibitors, suggesting combination of BRAF and MEK inhibition as a therapeutic approach for this subgroup of patients (Corcoran et al., 2010).

There are also studies on other rare somatic mutations in CRC patients. Among them, *POLE* (catalytic subunit of DNA polymerase epsilon) (Guerra et al., 2017) and *POLD1* (catalytic subunit of DNA polymerase delta1) are investigated as important contributors to ultramutation in CRC (Briggs and Tomlinson, 2013). Prognostic significance of *KDR* (Kinase insert Domain Receptor) in CRC patients is investigated in combination with the expression level of proteins such as VEGFA (Vascular Endothelial Growth Factor A) and FLT1 (a cell-surface receptor for VEGFA), pointing out the importance of thorough comprehension of somatic mutations in predicting response for CRC patients (Zhang et al., 2015).

Four of the most mutated genes in CRC (*TP53, APC, SMAD4, KRAS*) were disrupted using CRISPR-Cas9 technology in human intestinal stem cells to investigate the invasiveness of CRC cancer cells. Loss of both *TP53* and *APC* was shown to be sufficient for the emergence of aneuploidy and tumor progression, nevertheless only quadruple-mutant organoids could produce poorly differentiated, highly proliferative, and larger tumors in vivo (Drost et al., 2015).

9. Conclusion

NGS technologies constitute a recent revolution in science. However, correct functional interpretation of the identified genetic variants is nowadays the bottleneck for most studies to reach valid conclusions. Available advances have been described in this chapter and including bioinformatics, cell-based assays, CRISPR-Cas9, ZFN and TALEN systems, organoids and xenograft models, that will permit to move in this direction in the near future. Despite these important advances, the calibration of the calibration of the relative weight of each evidences for every gene and the standardization of functional tests are important challenges for the coming years.

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Appendix A. Supplementary data

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